Lasers in Surgery and Medicine 26:357–363 (2000)

# Laser Modulation of Angiogenic Factor Production by T-Lymphocytes

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***Background and Objective:*** **In previous investigations, small variations in the energy densities of low level light therapy (LLLT) were found to produce significant differences in the proliferation of resting T-lymphocytes in vitro. Pulsing these cells with mitogen in addition to laser therapy produced inhibitory effects regardless of the amplitude of the energy density used. In the current study, the effect of LLLT on the production of angiogenic factor(s) by T-lymphocytes was investigated in vitro.**
***Study Design/Materials and Methods:*** **Human T-cells isolated from peripheral blood were prepared in suspension either with or without addition of mitogen. Cell suspensions were irradiated with laser by using the following energy densities: 1.2, 3.6, 6.0, and 8.4 J/cm$^2$. Wavelength, pulsing frequency, and power output were kept constant at 820 nm, 5,000 Hz, and 50 mW, respectively. After either 3 or 5 days of incubation, lymphocyte supernatants were collected and added as conditioned media to cultured endothelial cells (ECs). The effect on the proliferation of these ECs was assessed over a 72-hour period by using a methylene blue assay.**
***Results:*** **Endothelial cell proliferation increased significantly when incubated with conditioned media collected from resting T-cells exposed to 1.2 and 3.6 J/cm$^2$. Day 5 conditioned media produced similar patterns of EC proliferation to that of day 3 but at lower magnitude. Pulsing of T-lymphocytes with mitogen in addition to laser irradiation significantly lessened their angiogenic capability. Conditioned media from 3.6 J/cm$^2$ laser-treated T-cells induced the maximal EC proliferation in all groups studied.**
***Conclusion:*** **It would seem that laser therapy stimulates lymphocytes to produce factor(s) that can modulate EC proliferation in vitro; this effect on the lymphocytes is influenced by (1) the amplitude of energy density used for T-cell irradiation, (2) exposing T-cells to both mitogen and laser, and (3) the duration of T-cell incubation in culture. Lasers Surg. Med. 26:357–363, 2000** **© 2000 Wiley-Liss, Inc.**

**Key words: light therapy (LLLT); in vitro; endothelial cells; proliferation; conditioned media; methylene blue assay; mitogen (PHA)**

## INTRODUCTION

Angiogenesis is an essential component of normal development, tissue repair, and tumour growth. It is necessary for wound healing [1], resulting in the formation of granulation tissue and the re-establishment of vascular integrity. Neovascularisation restores the supply of oxygen and nutrients to the new tissue and accommodates the high local metabolic demands and continued growth of the reparative cells permitting them to migrate, divide, and synthesise [2]. It also aids in the removal of waste products. The principal cell type of angiogenesis is the microvascular endo-

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Accepted 29 November 1999

thelial cell (EC) [3]. In response to appropriate stimuli, these cells undergo migration and proliferation; ultimately they form capillaries and line other vessels of the new microcirculatory bed. Although the mechanism of induction of angiogenesis is not fully understood, endothelial cell proliferation is considered to be an important prerequisite of the process of angiogenesis [4]. The factors that stimulate angiogenesis in wound repair are not fully known; however, many endothelial cell growth factors have been characterised by in vitro assays and many of these been recovered from macrophages [5].

The role of T-lymphocytes in wound healing has begun to be recognised [6]; however, their contribution to the angiogenic process requires further investigation. Lymphokine-containing supernatants have been found to enhance proliferation and chemokinesis of ECs [7]. Well-characterised lymphokines such as IFN-$\gamma$ [8,9], TGF-$\beta$ [10,11], vascular endothelial growth factor (VEGF) [12], and IL-4 [13] have been observed to regulate different functions of ECs in vitro. Recently, Mach et al. [14] reported that T-lymphocytes can activate ECs to synthesise matrix metalloproteinase by means of a CD40L-dependent mechanism. Furthermore, lymphocyte suspensions injected into irradiated abdominal skin of mice have been found to stimulate new vessel formation [15]. It was proposed that T-helper cells were responsible for the induction of angiogenesis through the release of a lymphokine designated as "lymphocyte-induced angiogenesis factor" (LIA). Monte et al. [16,17] demonstrated that T-lymphocytes from tumour-bearing mice can elicit in vivo a strong neovascular response in a process called syngeneic lymphocyte-induced angiogenesis (SLIA).

Advances in understanding of the role of angiogenesis in wound healing and in the pathology of different diseases have made this a prime target for therapeutic manipulation. Low-level laser therapy (LLLT) has been observed to enhance and to accelerate the formation of new vessels in rabbit ear chambers [18]. The process of neovascularisation in the skin flaps in hamsters [19] and in rats [20] was also stimulated in response to LLLT. In vitro, LLLT has been shown either to have a negligible effect [21] or to stimulate or inhibit the proliferation of bovine ECs, depending on the wavelength and energy density used [22].

The effect of LLLT on the proliferation of T-lymphocytes has been shown to be influenced by (a) the energy density amplitude and (b) the status of T-cell activation [23]. This study was designed to demonstrate the effect of LLLT on the production by T-lymphocytes of growth factor(s) that are capable of regulating the proliferation of ECs in vitro.

## MATERIALS AND METHODS

### Separation of T-Lymphocytes

Mononuclear cells (MNCs) were first separated by a density gradient method in which heparinised peripheral human blood was layered over Ficoll-Isopaque solution (HISTOPAQUE-1077) [Sigma] and centrifuged at 400 *g* for 30 minutes [24]. Monocytes were isolated from the MNCs by adherence to plastic [25]. After this, the rosetting technique [26] was applied for the separation of T-lymphocytes from the recovered nonadherent MNCs. The T-lymphocytes were then resuspended in RPMI 1640 (GIBCO) + 10% heat-inactivated autologous serum. Monocytes were added at a ratio of 6–8% to act as accessory cells for the induction of T-lymphocyte activation [27]. The cells were suspended at a concentration of $10^6$ cells/ml and then divided into two aliquots, to one of which purified mitogen (phytohaemagglutinin, or PHA) at a dose of 5–10 $\mu$g/ml, was added.

### Laser Irradiation and Dosimetry

One millilitre of cell suspension was transferred into each of the four corner wells of a 24-well plate immediately before irradiation with a Gallium-Aluminium-Arsenide laser device (Biotherapy 3ML - OMEGA, London, UK). A single irradiation of laser was applied to each well by using one of the following energy densities: 1.2, 3.6, 6.0, and 8.4 Joules/cm$^2$. The wavelength, pulsing frequency, and power output were kept constant at 820 nm, 5,000 Hz, and 50 mW, respectively. The control group was sham-irradiated. All samples were maintained in darkness in a 5% $CO_2$ humidified incubator. After either 3 or 5 days of incubation, the supernatants were collected, filtered, and then stored at –20°C.

### Primary Culture of Bovine Aortic EC

The method of Booyse et al. [28] for culturing endothelial cells from bovine aorta was slightly modified. The aorta was opened dorsally and scrapings from the inner surface were incubated in collagenase solution. The separated cells were washed with minimal essential medium (MEM) (GIBCO) containing antibiotics and then resus-

pended in MEM + 10% fetal calf serum (FCS) (GIBCO) and placed in a humidified incubator at 37°C supplied with 5% $CO_2$. Confluent EC monolayers at passage two were characterised by immunostaining with anti–factor-VIII–related antigen by using an immunofluorescent technique.

### EC Proliferation Assay

EC suspensions ($2 \times 10^4$ cells/ml) were prepared and 50 μl aliquots were placed in each well of 96-well microtitre plates. To every column of six wells, 50 μl of T-cell conditioned media from each of the irradiated groups, as well as the controls, were added. The plates were incubated for either 24, 48, or 72 hours at 37°C in a humidified incubator supplied with 5% $CO_2$. The methylene blue assay [29] was used for assessing EC proliferation. Absorbance at 650 nm, shown by Oliver et al. [29] to be linearly related to cell number, was measured with a spectrophotometer (Anthos 2001).

### Statistical Analysis

Differences between energy density levels at 72 hours in nonmitogen- and mitogen-pulsed groups were analysed by using two-way analysis of variance. Subsequent contrasts of absorbance between different energy densities and controls were performed by using Dunnett's test [30]. Differences between nonmitogen- and mitogen-pulsed groups at individual energy density levels were analysed by using the Tukey test [30].

## RESULTS

### Effect of Day 3 Conditioned Media of Laser-Irradiated T-Lymphocytes

ECs treated with conditioned media from resting lymphocytes exposed to energy densities of 1.2 and 3.6 J/cm$^2$ showed significantly higher absorbance values (of 650 nm light) than the control ($P < 0.05$) (Table 1; Fig. 1). Although the energy density of 6.0 J/cm$^2$ had no detected effect on ECs, 8.4 J/cm$^2$ produced significant inhibition ($P < 0.05$). In the group treated with conditioned media from mitogen-pulsed lymphocytes, the only positive effect was induced in response to the energy densities of 3.6 J/cm$^2$ ($P < 0.05$) (Table 2; Fig. 2). The absorbance readings for nonmitogen-pulsed group were significantly higher than those for mitogen-pulsed group at all energy density levels as well as controls ($P < 0.05$).

**TABLE 1. Effect of day 3 Condition Media of Laser-Irradiated Resting T-Lymphocytes**

| Energy density | Time postplating (hr) | Mean absorbance | SEM | Statistically different |
|---|---|---|---|---|
| Sham | 24 | 0.323 | 0.007 | |
| | 48 | 0.366 | 0.014 | |
| | 72 | 0.762 | 0.042 | |
| 1.2 | 24 | 0.368 | 0.009 | — |
| | 48 | 0.502 | 0.013 | — |
| | 72 | 0.924 | 0.012 | Yes |
| 3.6 | 24 | 0.365 | 0.003 | — |
| | 48 | 0.563 | 0.008 | — |
| | 72 | 0.932 | 0.012 | Yes |
| 6.0 | 24 | 0.351 | 0.005 | — |
| | 48 | 0.399 | 0.007 | — |
| | 72 | 0.690 | 0.014 | No |
| 8.4 | 24 | 0.327 | 0.004 | — |
| | 48 | 0.439 | 0.013 | — |
| | 72 | 0.599 | 0.044 | Yes |

**TABLE 2. Effect of Day 3 Condition Media of Laser-Irradiated and Mitogen-Pulsed T-Lymphocytes**

| Energy density | Time postplating (hr) | Mean absorbance | SEM | Statistically different |
|---|---|---|---|---|
| Sham | 24 | 0.310 | 0.004 | |
| | 48 | 0.339 | 0.004 | |
| | 72 | 0.376 | 0.011 | |
| 1.2 | 24 | 0.330 | 0.005 | — |
| | 48 | 0.406 | 0.010 | — |
| | 72 | 0.450 | 0.015 | No |
| 3.6 | 24 | 0.337 | 0.004 | — |
| | 48 | 0.459 | 0.007 | — |
| | 72 | 0.556 | 0.019 | Yes |
| 6.0 | 24 | 0.313 | 0.004 | — |
| | 48 | 0.339 | 0.006 | — |
| | 72 | 0.361 | 0.011 | No |
| 8.4 | 24 | 0.325 | 0.005 | — |
| | 48 | 0.345 | 0.006 | — |
| | 72 | 0.392 | 0.025 | No |

### Effect of Day 5 Conditioned Media of Laser-Irradiated T-Lymphocytes

The response of ECs to day 3 conditioned media was similar to their response to day 5 conditioned media but at lower magnitude (Tables 3 and 4; Figs. 3 and 4). ECs treated with conditioned media from resting lymphocytes exposed to energy densities of 1.2 and 3.6 J/cm$^2$ showed significantly higher absorbance values than the control ($P < 0.05$). The absorbance readings at 6.0 and 8.4 J/cm$^2$ were lower than the control. Similarly, in the group treated with conditioned media from mitogen-pulsed lymphocytes, there was a significantly higher absorbance value at 3.6 J/cm$^2$ than

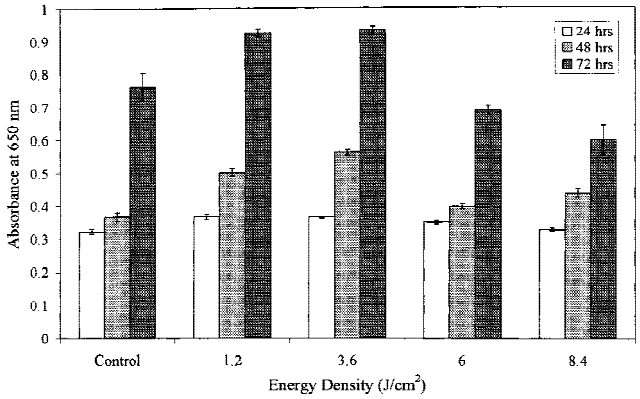


Fig. 1. The effect of day 3 condition media of laser-irradiated resting T-lymphocytes on EC proliferation in vitro.

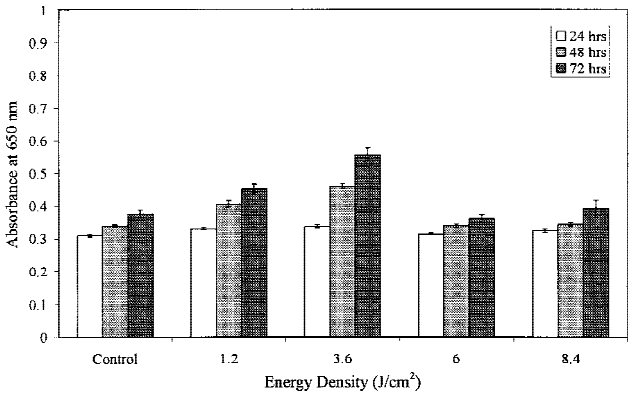


Fig. 2. The effect of day 3 condition media of laser-irradiated and mitogen-pulsed T-lymphocytes on EC proliferation in vitro.

the control ($P < 0.05$). However, there were lower absorbance values at the other energy densities of 1.2, 6.0, and 8.4 J/cm$^2$. The absorbance readings for the nonmitogen-pulsed group were higher than those for the mitogen-pulsed group at 1.2 and 3.6 J/cm$^2$ ($P < 0.05$).

The maximal proliferative activity of ECs, as indicated by the absorbance values, was produced by supernatants from lymphocytes exposed to LLLT at the energy density of 3.6 J/cm$^2$ in the all groups studied.

## DISCUSSION

The results described above show that light therapy is capable of stimulating endothelial cell proliferation indirectly by interacting with T-lymphocytes. In this work, the response of ECs to different conditioned media of laser-irradiated T-lymphocytes varied significantly both within and between groups. Although the lower energy densities of laser indirectly produced a stimulatory effect on ECs, the higher energy densities were inhibitory. At 72 hours after incubation, the absorbance values, indicative of EC proliferation, were significantly increased in all groups studied when incubated with conditioned media from lymphocytes irradiated at 3.6 J/cm$^2$. In contrast, proliferation of ECs was inhibited when incubated with conditioned media from T-cells irradiated with LLLT at energy densities of 6.0 and 8.4 J/cm$^2$.

T-lymphocytes have been shown to be extremely sensitive to light therapy in vitro [23]. Small variations in the amplitude of energy density resulted in broad differences in the proliferation of resting of T-cells. The lower energy densi-

**TABLE 3. Effect of Day 5 Condition Media of Laser-Irradiated Resting T-Lymphocytes**

| Energy density | Time postplating (hr) | Mean absorance | SEM | Statistically different |
|---|---|---|---|---|
| Sham | 24 | 0.316 | 0.003 | |
| | 48 | 0.349 | 0.005 | |
| | 72 | 0.405 | 0.003 | |
| 1.2 | 24 | 0.321 | 0.007 | — |
| | 48 | 0.426 | 0.008 | — |
| | 72 | 0.544 | 0.007 | Yes |
| 3.6 | 24 | 0.344 | 0.002 | — |
| | 48 | 0.463 | 0.015 | — |
| | 72 | 0.681 | 0.010 | Yes |
| 6.0 | 24 | 0.295 | 0.006 | — |
| | 48 | 0.348 | 0.013 | — |
| | 72 | 0.363 | 0.009 | Yes |
| 8.4 | 24 | 0.298 | 0.003 | — |
| | 48 | 0.346 | 0.006 | — |
| | 72 | 0.384 | 0.004 | No |

**TABLE 4. Effect of Day 5 Condition Media of Laser-Irradiated and Mitogen-Pulsed T-Lymphocytes**

| Energy density | Time postplating (hr) | Mean absorbance | SEM | Statistically different |
|---|---|---|---|---|
| Sham | 24 | 0.335 | 0.007 | |
| | 48 | 0.356 | 0.008 | |
| | 72 | 0.403 | 0.004 | |
| 1.2 | 24 | 0.339 | 0.007 | — |
| | 48 | 0.357 | 0.012 | — |
| | 72 | 0.373 | 0.005 | Yes |
| 3.6 | 24 | 0.366 | 0.010 | — |
| | 48 | 0.469 | 0.005 | — |
| | 72 | 0.647 | 0.012 | Yes |
| 6.0 | 24 | 0.344 | 0.008 | — |
| | 48 | 0.362 | 0.003 | — |
| | 72 | 0.392 | 0.003 | No |
| 8.4 | 24 | 0.326 | 0.005 | — |
| | 48 | 0.345 | 0.008 | — |
| | 72 | 0.399 | 0.009 | No |

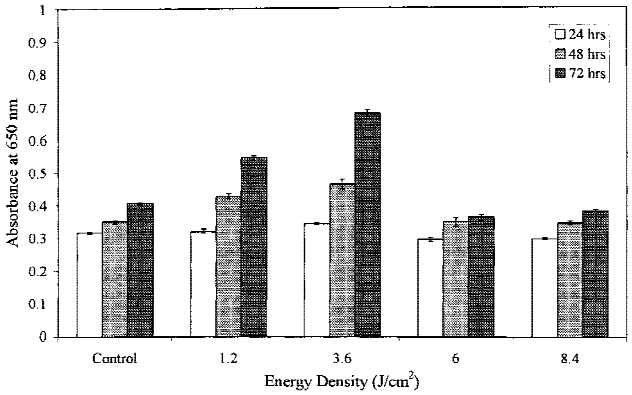


Fig. 3. The effect of day 5 condition media of laser-irradiated resting T-lymphocytes on EC proliferation in vitro.

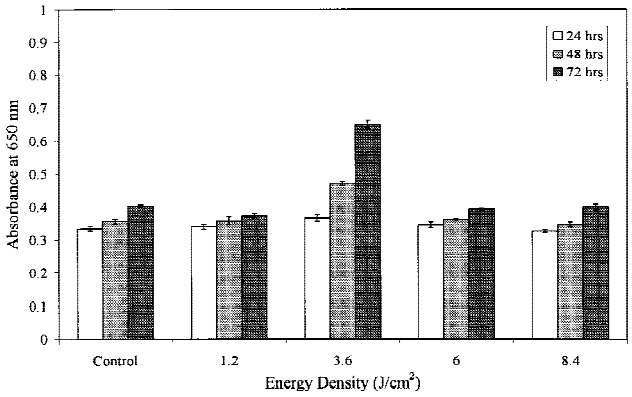


Fig. 4. The effect of day 5 condition media of laser-irradiated and mitogen-pulsed T-lymphocytes on EC proliferation in vitro.

ties produced stimulatory effects on resting T-lymphocytes with a peak at 3.6 J/cm$^2$, and the higher energy densities produced inhibitory effects. Based on this, the current work was designed to examine the effect of LLLT on angiogenic factor production by T-lymphocytes by using similar laser parameters. The outcome of the nonmitogen treated groups in both investigations was closely related and supports the hypothesis that LLLT has a dose-related effect in modulating cell functions [31,32]. The current work also demonstrated the high sensitivity of immune cells to light therapy and echoes Ohshiro and Calderhead's graphic interpretation of the Arndt-Schultz law [33].

Previously, it has been shown that laser therapy can induce inhibition of proliferation in mitogen-pulsed T-lymphocytes [34,35], regardless of the energy density used [23]. In the current work, the production of angiogenic factors was significantly lowered in the mitogen-pulsed groups compared with the nonmitogen-pulsed groups as judged by the proliferation of ECs. Mitogens are known to increase the proliferation and the secretion of active products by T-lymphocytes [36]. Likewise, laser, applied at certain energy levels, has been shown to stimulate proliferation and growth factor production by these immune cells [23,32]. Previously published studies and the current work have shown that combining both mitogen and laser treatment has a negative effect on T-cell activity. The explanation for this is not known; however, both mitogen [36] and LLLT [37] induce rapid influx of calcium into the cells. This could lead to increase in the accumulation of intracellular calcium and, if excessive, consequently to T-cell death [38].

Hadar et al. [15] speculated that angiogenic factor production by T-lymphocytes is dependent qualitatively more on the level of lymphocyte function rather than on the cell number. Laser therapy has been shown to inhibit the proliferation of mitogen stimulated T-cells. However, in the current study, the production of angiogenic factor(s) by laser treated lymphocytes was not affected in the same way, as certain low energy densities produced a stimulatory effect on EC proliferation. Although it is possible that cell damage or cell death occurred in the groups treated with both laser and mitogen, it is also possible that the surviving cells were able to respond sufficiently to produce angiogenic factor(s) and, thus, to sustain some proliferative effects on ECs.

The literature indicates that light photobioactivation accelerates wound healing [39,40] and promotes angiogenesis [41]. Although the way lasers exert this effect is not fully understood, enhanced wound healing must be caused by changes at a cellular level. In vitro light therapy has been found to modulate the activity of a variety of cells involved in the process of wound healing including macrophages, fibroblasts, keratinocytes, mast cells, lymphocytes, and ECs (reviewed by Baxter) [42]. Most in vitro effects of LLLT have been reported in relation to the proliferation of single cell types; however, the in vivo situation is different in that the healing process involves a complex interaction of cells. Immuno-inflammatory cells such as macrophages and T-lymphocytes are capable of producing cytokines and growth factors that control the functions of other cells and regulate the processes of angiogenesis and fibroplasia. Laser-irradiated macrophages have been found to produce conditioned media that contain growth factors able to modulate the proliferation of fibroblasts

[43,44] and ECs (unpublished data). In this study, the dose response of ECs to conditioned media of laser-irradiated T-lymphocytes was closely similar to their response to the conditioned media of laser-irradiated macrophages. By using the same laser parameters, the production of angiogenic factor by macrophages was stimulated at 2 J/cm$^2$ and inhibited by the higher energy density of 8 J/cm$^2$.

Collectively, the results obtained show that there is a dose-dependent response of the synthesis and secretion of angiogenic factor(s) by T-lymphocytes in response to light therapy, exhibited in the modulation of EC proliferation. Angiogenic factor production was influenced by the energy density amplitude, the addition of mitogen to T-lymphocytes, and the duration of their incubation in culture. The angiogenic factor(s) produced by the T-lymphocytes, have yet to be identified.

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